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Role of Tonic Protein Kinase A on SK2 Channel Expression

Krithika Abiraman

B.Tech. Anna University, 2010

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

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APPROVAL PAGE

Master of Science Thesis

Role of Tonic Protein Kinase A on SK2 Channel Expression

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University of Connecticut

2012

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ABSTRACT

Small conductance calcium-activated potassium channels (SK) play a fundamental role in synaptic transmission, plasticity, learning and memory (Hammond, 2006; Faber, 2008; Lin et al., 2008) . A very recent study successfully combined single molecule atomic force microscopy (AFM) and toxin pharmacology to quantitatively map SK channels in living neurons (Maciaszek et al., 2012). In addition to localizing native SK channels, this study also showed that SK channel distribution is dynamic with a decrease in SK channel surface expression upon addition of forskolin (FSK), a protein kinase A (PKA) activator. This finding raised further questions about the mechanism through which FSK acts to reduce SK channel expression. In the present work, it was found that FSK acts via the cyclic adenosine-dependent PKA pathway. Also, while PKA activator (FSK) decreased SK channel surface expression, PKA inhibitor (KT 5720) caused the opposite effect. This suggests that there is a tonic PKA activity which suppresses the surface expression of SK channels. Experiments were first carried out in heterologous expression system, HEK293T cells expressing SK channels and then in rat hippocampal neurons expressing native SK channels.

Chapter 1. INTRODUCTION

The combination of toxin pharmacology and single molecule atomic force microscopy (AFM) has been very recently employed to quantitatively map small conductance calcium-activated potassium channels (SK) in living neurons (Maciaszek et al., 2012). This study showed that the SK channels are highly concentrated in the neuronal dendrites and are organized on the neuronal membranes as single entities or groups of two. It was also found that the distribution of the SK channels in the rat hippocampal neurons is dynamic which showed a decrease in the surface expression of these channels upon addition of forskolin (FSK), a protein kinase A (PKA) activator. This study provided a method to detect SK channels in living neurons at a nanometer scale and the initial results that FSK decreases SK channel surface expression raised further questions about the pathway through which it acts. Other studies have shown that the cyclic adenosine monophosphate (cAMP)-dependent PKA pathway decreases the surface expression of the SK channels (Yajun Ren, 2006; Faber, 2008; Lin et al., 2008).

In order to address the mechanism of FSK action, the following study was carried out using same technique of integrating toxin pharmacology with single molecule AFM, in the presence of various neuromodulators. The quantitative effect of PKA was first studied in a heterologous expression system, HEK293T cells expressing SK channels and then in rat hippocampal neurons expressing native SK channels. The present work showed that the FSK indeed acts through the cAMP-

dependent PKA pathway and that there is a tonic PKA activity which suppresses the SK channel surface expression.

cAMP is a second messenger that regulates many cellular functions through the activation of PKA. Although cAMP has several other downstream effectors like cyclic nucleotide-gated cation channels and a family of guanine nucleotide exchange factors (TASKÉN and AANDAHL, 2004), PKA is generally recognized as its primary effector. Previous works studying the effect of cAMP-dependent PKA in SK channel expression relied on molecular techniques like immunohistochemistry - specifically immunofluorescence - which suffer from major drawbacks like sample fixation and non-specific binding. Fixation of the sample by chemical means could alter the binding properties of the sample and could also affect antigen recognition by the antibody. Also sample fixation impairs the ability to do dynamic studies. The presence of non-specific binding forces in antibody-antigen systems usually produces a large background effect obscuring the detection of the target antigens. To overcome these limitations, we combined single molecule atomic force microscopy and toxin pharmacology to detect the quantitative effect of PKA and the different components of the PKA pathway on SK channel surface expression.

Chapter 2. REVIEW OF LITERATURE:

2.1 Small conductance calcium-activated potassium channels

Calcium-activated potassium channels are a family of potassium channels that are distinct from the voltage-gated potassium channels. These channels are voltage-independent and are gated solely by intracellular calcium. Activation of calcium-activated potassium current was first demonstrated in erythrocytes where they caused membrane hyperpolarization and cell shrinkage (Gárdos, 1958). Later by pharmacological manipulation of cytosolic calcium, calcium-activated potassium currents were observed in mollusc neurons (Meech, 1972) and in cat spinal motor neurons (Krnjević, 1972). These currents were shown to underlie the afterhyperpolarization (AHP) that regulates action potential firing in hippocampal CA1-CA3 pyramidal neurons. Subsequently, based on molecular and pharmacological properties, three types of calcium-activated potassium channels were described: large conductance (BK), intermediate conductance (IK) and small conductance (SK) calcium-activated potassium channels. In this study we focus on small conductance calcium activated potassium channels (SK).

The SK channels are called so because of their relatively small single channel conductance of approximately 10pS (Hirschberg, 1999) compared to BK channels that have single channel conductance of 100-200pS. The SK channel family consists of three subtypes (SK1-3) and they display partially overlapping but distinct distributions. SK1 and SK2 are expressed at their highest densities in hippocampus and cortex whereas SK3 subunits are expressed at highest levels

in hypothalamus, thalamus and midbrain (Stocker and Pedarzani, 2000; Sailer et al., 2002; Sailer et al., 2004).

SK channels are blocked by a variety of pharmacological agents like peptide toxins such as apamin (Blatz and Magleby, 1986) and scyllatoxin (Weatherall et al., 2010), organic compounds like D-tubocurarine , quaternary salts of bicuculline and cyclophane derivatives like UCL1684 (Adelman et al., 2012). Scorpion toxin, tamapin and an analog of scyllatoxin – leiurotoxin-Dab selectively block SK2 channels(Weatherall et al., 2010). Certain compounds like 1-EBIO, CyPPA and NS309 enhance SK channel activity by increasing their calcium sensitivity (Hougaard et al., 2007).

Bee venom toxin apamin, is the most commonly used SK channel blocker. It is unique in its action that it displays selectivity among the SK channel subtypes. The SK2 channel is the most sensitive to apamin (half maximal inhibitory concentration i.e. the effectiveness of apamin in inhibiting the SK2 channels, IC_{50} 0.03-0.14nM) followed by SK3 (IC_{50} 0.6-4nM) (Takahiro M. Ishii, 1997) and human SK1 is the least sensitive (IC_{50} 0.1-12nM) (M Shah, 2000) and rat SK1 being insensitive to apamin. The difference in the sensitivity to apamin between hSK1 and SK2 channels is due a “serine-threonine switch” i.e. the replacement of serine residue with threonine on the extracellular region between S3 and S4 on the hSK1 subunit (Nolting et al., 2007).

2.1.1 Activation by calcium

SK channels are gated by calcium (Ca^{2+}), a ubiquitous second messenger whose cytosolic concentration is controlled by buffers, calcium pumps and transporters (Carafoli, 1987). Intracellular Ca^{2+} ions orchestrate a wide variety of Ca^{2+}-dependent signaling and reaction cascades, for which it is important that the Ca^{2+} signal is precisely located in time and space. A variety of Ca^{2+} buffer systems restrict the diffusion of Ca^{2+}, after they have entered the cell through Cav channels, to "local Ca^{2+} signaling domains" (Augustine et al., 2003). These domains are classified as either Ca^{2+} nanodomains (within ~20-50nm of the Ca^{2+} source) or Ca^{2+} microdomains (distance between 50 to a few hundred nm from the Ca^{2+} source) (Augustine et al., 2003). SK channels most likely exist within a microdomain of a Ca^{2+} source that provides Ca^{2+} for its activation (Fakler and Adelman, 2008).

Although the SK channels are gated by $Ca^{2+,}$ they do not have an intrinsic Ca^{2+} binding domain. Instead they are constitutively bound to calmodulin (CaM) which in turn binds to Ca^{2+} (Xia et al., 1998). CaM binds to each subunit of the tetrameric channel immediately after the sixth transmembrane domain called the calmodulin binding domain (CaMBD) which is highly conserved across the SK family (Fig 2.1.2). Binding of Ca^{2+} CaM, causes a conformational change in the SK channel that results in channel opening while Ca^{2+} unbinding causes the opposite effect (Keen, 1999). The structure of CaMBD in complex with CaM-Ca^{2+} is a dimer of two CaMBDs and two CaMs occupied with Ca^{2+} ions. In the absence of Ca^{2+} the CaMBD-CaM complex is monomeric and extended.

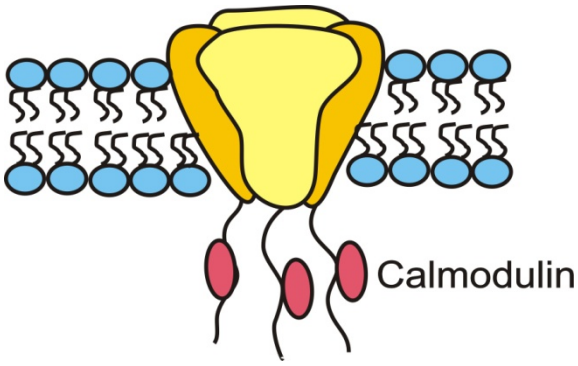

Figure 2.1.1 Small conductance calcium activated potassium channels: Schematic of SK channels with tetrameric pore-forming subunits with constitutively bound calmodulin.

Apart from CaM, protein kinase CK2 and protein phosphatase 2A (PP2A) are also constitutively bound components of SK channels and finely regulate the Ca^{2+} sensitivity of the SK channels (Bildl et al., 2004; Allen, 2007). The CK2 and PP2A have opposite regulatory effects on SK channels. CK2 phosphorylates the SK-bound CaM and decreases the Ca^{2+} sensitivity (from a half maximal effective concentration EC_{50} of ~0.5 µM to an EC_{50} of ~2 µM) (Bildl et al., 2004; Allen, 2007) of the channels and reduces the Hill coefficient (degree of cooperative binding of ligand to receptor) from 4 to 2. On the other hand, the PP2A dephosphorylates the SK-bound CaM and increases the Ca^{2+} sensitivity of the channels and increases the Hill coefficient to >4. Structure-function studies show that CK2 contacts multiple domains on the intracellular termini of the pore-

forming subunits and the PP2A is bound by a discrete domain just C-terminal to the CaMBD.

2.1.2 Functions of SK channels

In many neurons bursts of action potentials (AP) are followed by prolonged afterhyperpolarizarion (AHP) that may depend on Ca^{2+} influx (Barrett and Barret, 1976). The AHP has three phases: fast, medium and slow AHP (Sah, 1996). The fast AHP that overlaps the repolarization phase of the action potential is the initial component of the AHP and is mediated by BK channels and some voltage gated potassium channels. It typically lasts for 10-20 ms and contributes to spike repolarization (Storm, 1987; Gu et al., 2007). The slow AHP first described in myenteric plexus (Sah and Louise Faber, 2002) can last several seconds however the channels that underlie the slow AHP still remain unclear. The medium AHP (mAHP) is blocked by apamin thus indicating that this component of the AHP is due to SK channel activation. The SK channels have been shown to underlie the mAHP in a wide variety of neurons like spinal motorneurons (Zhang and Krnjević, 1987), pyramidal neurons in sensory cortex (Sah and McLachlan, 1992), lateral and basolateral amygdale (Power and Sah, 2008) and cerebellar Purkinje neurons (Womack and Khodakhah, 2003). The contribution of SK channels in AHP is largely due Ca^{2+} influx through voltage-gated Ca^{2+} (Cav) channels. The medium AHP controls the firing patterns of neurons and thereby regulates the frequency of action potential discharge. .

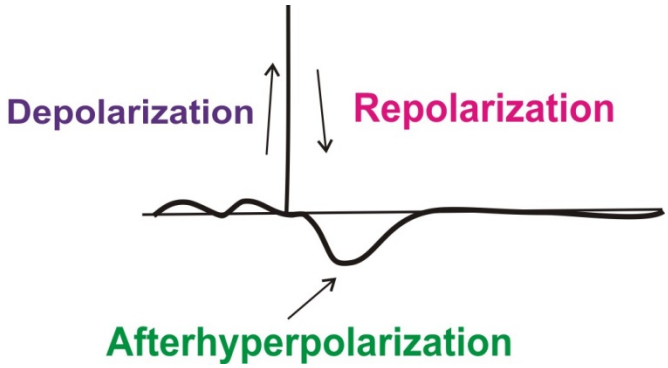

Figure 2.1.2: Action potential: Action potential with its three phases, depolarization, repolarization and afterhyperpolarization (AHP). The SK channels underlie the medium AHP (not shown).

Changes in SK channel expression contribute to memory and learning. The first such study reported that systemic injection of apamin accelerated memory acquisition (Messier et al., 1991). SK channel block also facilitates memory encoding and improves performance on a new object recognition task (Deschaux et al., 1997). Blockage of SK channels facilitates hippocampus-dependent spatial memory encoding. Further, apamin has also been shown to induce expression of c-fos and c-jun genes that are thought to be initial markers for memory formation (Heurteaux et al., 1993). Blockage of SK channels with apamin has been shown to facilitate learning in a number of behavioral studies. As opposed to the effects of blocking SK channels with apamin, increasing SK channel activity impairs learning. Overexpression of SK channels suppresses memory formation in amygdala and hippocampus-depenedent tasks suggesting that these channels regulate synaptic transmission and plasticity in lateral amygdala. In conclusion,

these studies show that SK channels play a key role in negatively regulating learning and memory formation, suggesting that SK channels may be suitable therapeutic targets in learning and memory disorders.

2.1.3 Neuromodulation of SK channels

Neuromodulators regulate firing properties of many neurons via secondary messengers that phosphorylate ion channels. Phosphorylation causes immediate or long term changes on channel properties and SK channels are important targets for phosphorylation as they regulate neuron firing. The C terminus region of the SK channels consists of a number of sites for phosphorylation (Yajun Ren, 2006). Studies in COS cells have shown that phosphorylation of serine residues within c-terminal domain of SK2 channels leads to its decrease in plasma membrane expression in COS cells (Yajun Ren, 2006). It was suggested that the SK2 were entrapped in the endoplasmic reticulum in their phosphorylated state(Yajun Ren, 2006). Regulation of SK channels has also been demonstrated in neurons. In pyramidal neurons, the activation of β adrenergic receptors causes the activation of adenylate cyclase and protein kinase A (PKA) leading to the reduction in SK channel expression and enhancement of excitatory post synaptic potentials (EPSPs) (Faber, 2008). Inductance of long-term potentiation (LTP) via PKA and N-Methyl-D-aspartic acid (NMDA) receptor activity has also been proved to reduce the number of SK channels expressed in postsynaptic density(Lin et al., 2008). From the above studies it is shown that PKA has a direct action on SK channels which reduces its function and expression. PKA is a serine threonine kinase that is dependent on cyclic adenosine monophosphate

(cAMP). Through the phosphorylation of target proteins, PKA regulates many biochemical processes.

The effect of PKA on SK channel expression has been previously studied using molecular techniques like immunohistochemistry. The major drawback of immunohistochemistry is that the cell sample is fixed which could alter its binding capacity. Also, the presence of non-specific binding forces in antibody-antigen systems usually produces a large background effect obscuring the detection of the target antigens. Atomic force microscopy eliminates the need for cell fixation thus ensuring that the binding properties are intact. Here, we examine the effect of cAMP-dependent PKA on SK channel expression using atomic force microscopy.

2.2. The cAMP-PKA pathway

The cAMP-PKA pathway is ubiquitously expressed and regulates a wide range of cellular functions. Various extracellular signals through the binding of ligand to G protein coupled receptor (GPCRs) tightly regulate the cAMP-PKA pathway and thereby helps maintain specificity. The GPCRs through the G proteins regulates adenylyl cyclase (AC) which leads to generation of cAMP. Adenylyl cyclase and phosphodiesterases (PDE) regulate the generation and degradation of cAMP respectively (Sunahara et al., 1996; Soderling and Beavo, 2000). There are several downstream effectors of cAMP the most common one being PKA.

The cAMP-dependent PKA is a heterotetramer consisting of two regulatory (RIα, RIβ, RIIα, RIIβ) and two catalytic subunits (C_α, C_β, C_γ) (Francis and Corbin, 1994). The binding of cAMP to the regulatory subunits, leads to the activation of the catalytic subunits. The active catalytic subunit then phosphorylates the target protein at the serine/threonine residues.

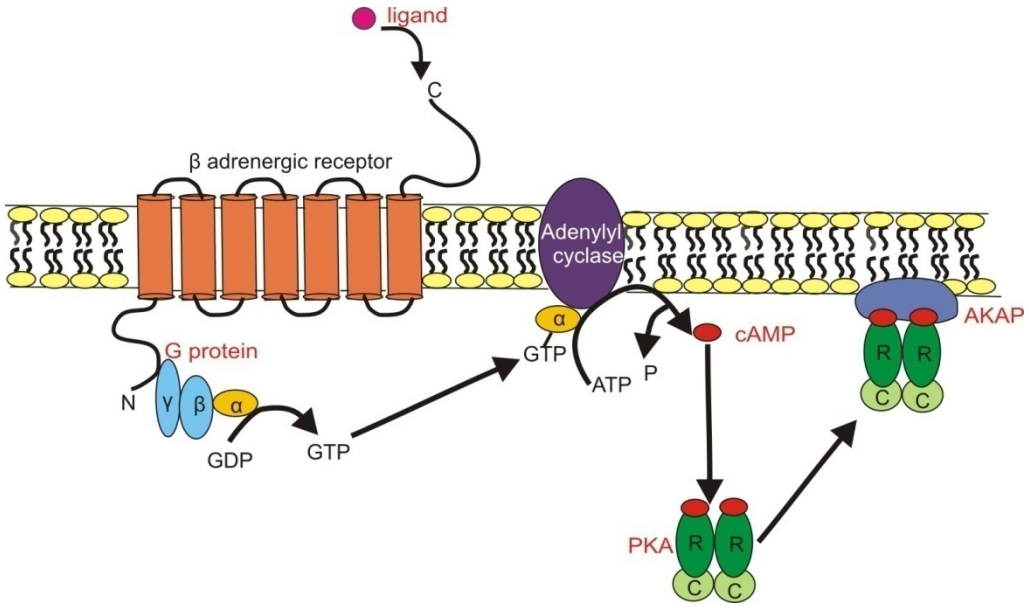

Figure 2.2.1 Canonical G_s-PKA pathway: Signal pathway depicting activation of G protein coupled receptors which then activates Adenylyl cyclase (AC) through Gα proteins. The activation of AC causes the production of cAMP which in turn activates the PKA holoenzyme. AKAP attaches to the PKA and localizes it to the plasma membrane. The plasma membrane localized PKA then phosphorylates its downstream effectors.

Given multiple PKA targets within distinct subcellular regions, a spatially confined PKA is essential to warrant its specific effects on targets. This is mainly achieved by A kinase anchoring proteins (AKAPs) (Colledge and Scott, 1999). Originally thought to be contaminants of purified PKA, it was later found that AKAPs enhance the efficiency and specificity of signaling events. AKAPs target PKA to specific substrates and subcellular compartments thereby rendering spatial and temporal specificity. The first demonstration that AKAP-mediated targeting of PKA is necessary for the mediation of a biological effect of cAMP was shown by peptide mediated disruption of PKA-AKAP complex directing PKA towards α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and the AKAP associated with PKA targeting to the AMPA receptor was later identified as AKAP79 (Rosenmund et al., 1994).

2.3. Atomic Force Microscopy

Atomic force microscope (AFM)(Binnig et al., 1986) initially designed for topographic imaging is now a powerful tool to manipulate biological systems in near physiological conditions. Its ability to work in liquid environments has opened a myriad of new applications in biology. Interactions of biomolecules play major roles in all biological processes and hence an understanding of these interactions would provide insights into these processes. AFM allows for the measurement of interactive forces at a single molecule level. This technique, called single molecule force spectroscopy (SMFS), enables the measurement of interactive forces down to pN range and with high spatial resolution (nanometer-scale). Using this method, a range of ligand-receptor interactions has been studied e.g., between biotin-avidin (Florin et al., 1994; Lee et al., 1994), antigen-antibodies (Hinterdorfer et al., 1996; Ros et al., 1998; Berquand et al., 2005), cadherins (Baumgartner et al., 2000), and intergrins (Zhang et al., 2002) .

AFM works by scanning a sharp tip over the sample in a raster manner. The nanometer-sharp tip is mounted at the end of a cantilever which deflects as it comes in contact with the sample. This deflection can be monitored using a laser beam that bounces off the back of the cantilever onto a position sensitive photodiode (PSD). The PSD is often connected to a feedback circuit that is employed to maintain a constant force between the tip and sample by adjusting the tip-sample distance.

AFM can accurately measure forces from piconewton to nanonewton scale. In order to study interactive forces using SMFS, the cantilever tip is attached to a ligand that can recognize a specific type of target molecule in the sample thereby enabling the detection of specific interactions at single molecule level. For biological samples, the tip used must have low spring constants ($<0.1 Nm^{-1}$). The AFM tip can be coated with a variety of molecules like antibodies and proteins. The tip, usually made of silicon or silicon nitride (Si_3N_4), is functionalized with a ligand via a linker like polyethylene glycol (Hinterdorfer et al., 1996). One end of the linker attaches to the Si_3N_4 tip and the other end is used to attach to the ligand of interest. The tips are appropriately functionalized so that the forces between the tip and the ligand are stronger than the forces between the ligand and the receptor in the sample so that the ligand does not detach from the probe. Also, the ligand is attached to the tip at low surface density to ensure single-molecule detection.

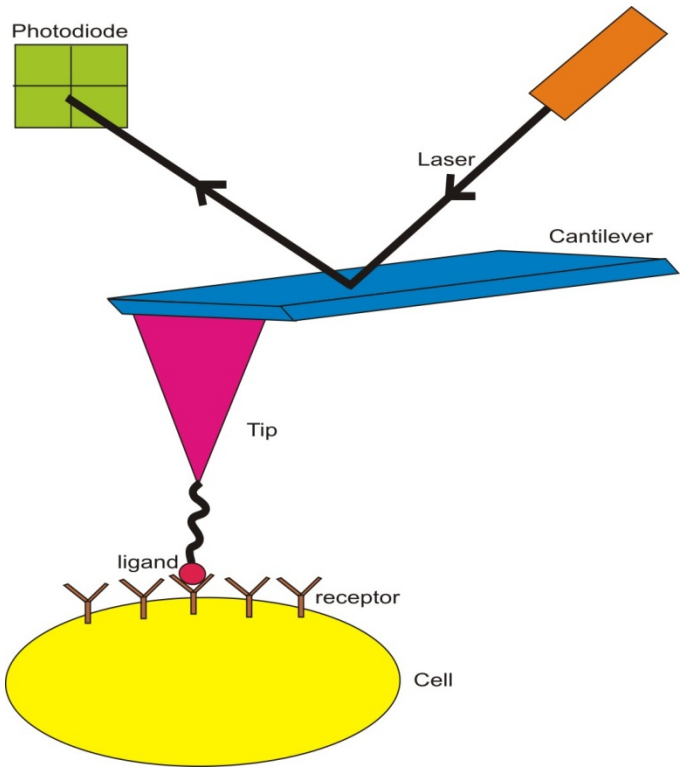

Figure 2.3.1 Schematic of functionalized tip attached to a cell surface receptor. The laser beam bounces off the back of the cantilever onto a photodiode which then maintains a constant force between the tip and sample by adjusting the tip-sample distance. As the ligand functionalized tip approaches the cell surface receptor, interaction between the ligand and receptor forms bonds which break upon retraction

The functionalized tip continuously advances and retracts from the biological sample, causing interactions between the functionalized tip and the receptor on the sample. These interactions in the form of cantilever deflection are recorded as force-distance curves. The force curves encompass an approach curve and retraction curve where the retraction component gives us information on the adhesion force between the functionalized tip and the receptor on the sample.

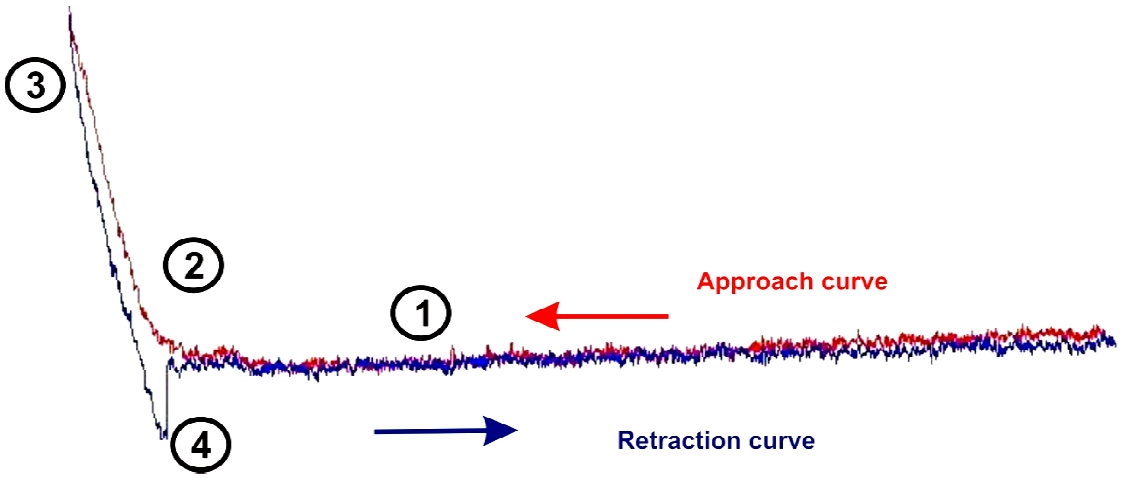

Figure 2.3.2 Force-distance curve: Force-distance curve obtained using Asylum MFP 3D-BIO (Asylum Research, Santa Barbara). The red curve is the approach curve and the blue curve is the retraction curve. Region 1 is the cantilever tip approaching the sample. Region 2 is the deflection of the cantilever tip as it comes in contact with the sample. Region 3 is the retraction of the cantilever tip from the sample. Region 4 is the negative deflection of the cantilever due to binding forces between the cantilever tip and the sample. As the cantilever restoring force overcomes the binding forces, the bond breaks leading to rupture forces. Rupture forces are used to measure the bond magnitude.

When the functionalized tip approaches the sample and it finds its complementary receptor, bonds are formed between the ligand on the tip and the receptor. These bonds break when the tip retracts from the surface of the sample thus forming rupture forces. The rupture forces can be visualized on the retraction curves. If the ligand on the tip does not form a bond, it implies that the recognition event is missing and the retraction curve looks like an approach

curve. The interaction force is proportional to the deflection of the cantilever following Hooke's law: $F = -k*d$, where F is the force, k is the spring constant of the cantilever and d is the displacement of the cantilever. The specificity of the interaction is established by blocking the receptor with free ligand and by using non-functionalized tips. Both cases show lack of rupture forces.

Chapter 3. MATERIALS AND METHODS

3.1. Transfection of SK2 channels in HEK293T cells

HEK293T cells were cultured and transfected with recombinant DNA (1.5 and 3.0 µg) using Lipofectamine 2000 (Invitrogen). SK2-S subcloned into a pEGFP-N1 vector (Clontech) was a gift from Chul-Seung Park, PhD (Gwangju Institute of Science and Technology, Gwangju, Korea) while SK2-L was a gift from John P. Adelman, PhD (Vollum Institute, OHSU).

3.2. Primary culture and fixation of rat hippocampal neurons

Rat hippocampal neurons were cultured in the Walikonis lab (Physiology & Neurobiology, University of Connecticut). Dissociated hippocampal cells from Sprague-Dawley rat pups on embryonic day 18 (E18) or E19 were plated onto 12-mm diameter poly-l-lysine/laminin-coated glass coverslips at a density of ~150 neurons/mm^2 as described in (Brewer et al., 1993; Lim and Walikonis, 2008) and grown in astrocyte-conditioned neurobasal media as described in (Goslin et al., 1998; Lim and Walikonis, 2008).

3.3. Preparation of AFM cantilever probes

Silicon nitride cantilevers were gently rinsed with ethanol and 18 MΩ water before being placed in a clean, dry petri dish with 30 µl APTES (3-aminopropy-triethoxysilane) and 10 µl triethylamine for 1h. N_3-dPEG-NH_2 linker was prepared by adding dry methylene choride (0.5 ml), triethylamine (7 µl), and 7.5 mg (7.5 µl) of N_3-dPEG-NH_2 linker (Quanta Biodesign, Powell, OH) to a small, dry glass vial.

The vial was sealed and inverted several times until all of the solid substances dissolved. After the probes were tightly sealed with APTES for 1h, they were removed and immediately added to the N_3-dPEG-NH_2 linker at 4℃. After 1h, the probes were rinsed with methylene chloride, ethanol, and then 18 MΩ water. The probes were placed in a parafilm-coated dish with their tips pointed upwards and inwards in a circular manner. 50 µl of 100 nM apamin is added to the center of the cantilevers, in contact with the probes. After 2 hours, AFM probes were rinsed with PBS buffer. If not used immediately the probes are stored in PBS buffer at 4℃ for use within 2-3 days of preparation.

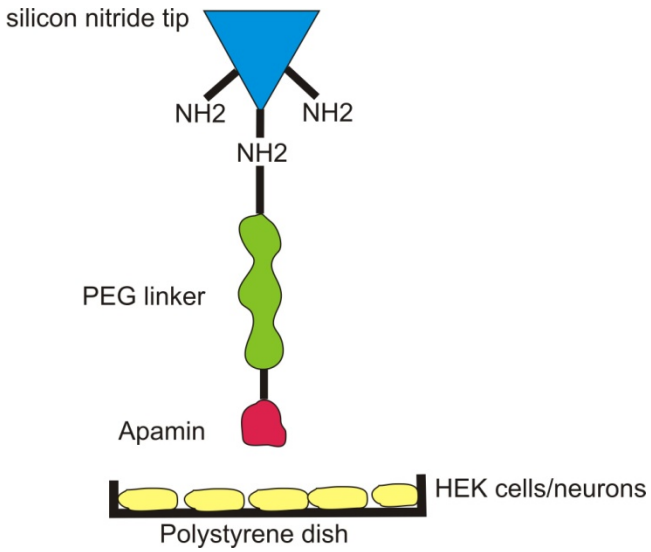

Figure 3.2.1 functionalized AFM tip. The silicon nitride tip is terminated with NH2 groups. It is then attached to a polyethylene glycol (PEG) linker whose other end is attached to bee venom toxin apamin.

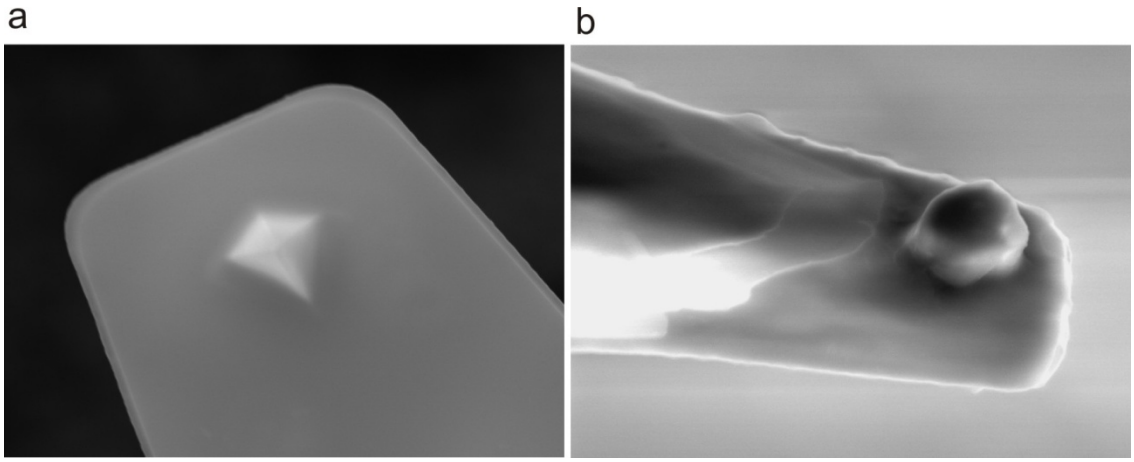

Figure 3.2.2 Scanning electron microscope (SEM) image AFM probe: (a) SEM (FEI Quanta 250 FEG) image of a non-functionalized probe. *Image was taken by Jing Qi (Center for Clean Energy Engineering, University of Connecticut)* (b) SEM (FEI Strata 400S DualBeam) image of apamin functionalized AFM probe. *Image was taken by Roger Ristau (Institute of Material Science, University of Connecticut)*

3.4. Atomic force microscopy measurements

Atomic force microscopy binding force maps and force-distance curves are obtained using an Asylum MFP 3D-BIO (Asylum Research, Santa Barbara, CA) AFM. HEK293T cell measurements are performed in Dulbecco's modified eagle media with 10% FBS and 1% panstrepavidin at 37℃.

All force measurements were carried out at a loading rate of 24000 pN/s. The cantilevers used had a nominal spring constant of 30pN/nm as provided by the manufacturer, the exact values of which are obtained via a thermal noise based

method implemented by the manufacturer. The approach and retraction velocities are held constant at 800 nm/s. Probes had nominal tip radii of 20 nm and nominal angle of 20°, as provided by the manufacturer.

3.5. Epifluorescence imaging

Epifluorescence optical imaging is employed to validate the transfection of SK2-S and SK2-L channels in the HEK293T cells. The inverted microscope attached to the AFM is equipped with fluorescence which allows for simultaneous validation of AFM images of surface SK channel distribution with epifluorescence.

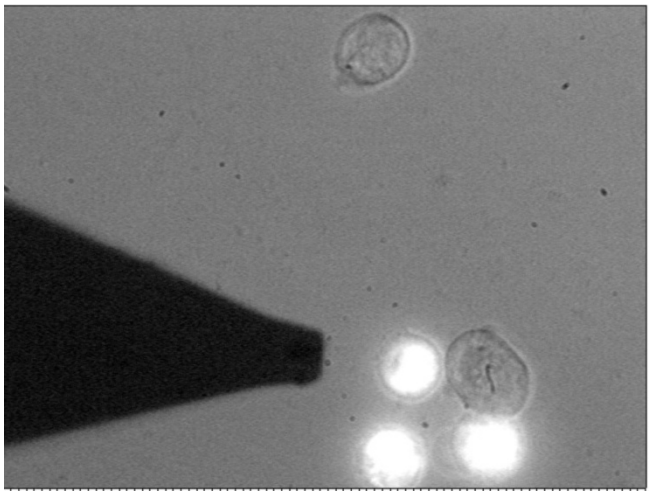

Figure 3.5.1 DIC image of SK2 channel-tranfected HEK293T cells: representative image of SK2 transfected HEK293T cells (bright cells) and untranfected HEK293T cells. Also seen is the AFM cantilever.

3.6. Data processing and analysis

On order to detect the distribution of SK2 channels on the cell surface, spatially-resolved binding force maps are recorded using an apamin functionalized probe

over 1 μm^2 areas. Binding forces are extracted from each of the 1024 force curves generated from each test. A MATLAB (Mathworks) program developed in our lab is then used to create visual binding force maps where each pixel represents an independent rupture force obtained from the retraction portion of the force-distance curve. To obtain the mean and variance of the bond magnitude of the apamin SK2 interactions for each experiment, the rupture forces obtained from the retraction portion of the force-distance curve were fit with a Gaussian distribution in MATLAB.

Chapter 4. RESULTS

4.1 SK2-S

In order to study the effect of PKA on SK2 channel activity we first studied the effect in a HEK293T cells. The specificity of apamin functionalized tips to SK channels have been previously demonstrated (Maciaszek et al., 2012). In order to study the effect of PKA on SK channels, binding events between the apamin functionalized tips and SK channels were studied in the presence of activators and inhibitors of PKA and the components of the PKA pathway. Rupture forces would be higher when the apamin functionalized tips come in contact with the SK channels than when it comes in contact with an area without any SK channel. Binding forces for each recording were done over 1μm^2 area of HEK293T cells transfected with SK and 1024 force-distance curves were generated.

First, baseline experiments were performed between apamin functionalized tips and SK2-S (3µg and 1.5µg) transected HEK293T cells. Binding events were observed in 4% of the sampled sites (3µg and 1.5µg) with similar mean binding forces (3µg, µ=25±5 pN and 1.5µg, µ=25±6 pN) (Fig 4.1.1). By plotting a histogram of the adhesion forces, a unimodal Gaussian distribution is obtained for both concentrations. The unimodal distribution along with the unchanged mean binding force indicates that there is a single population of SK2-S channels with no more than one SK2-S channel per 31.25nm.

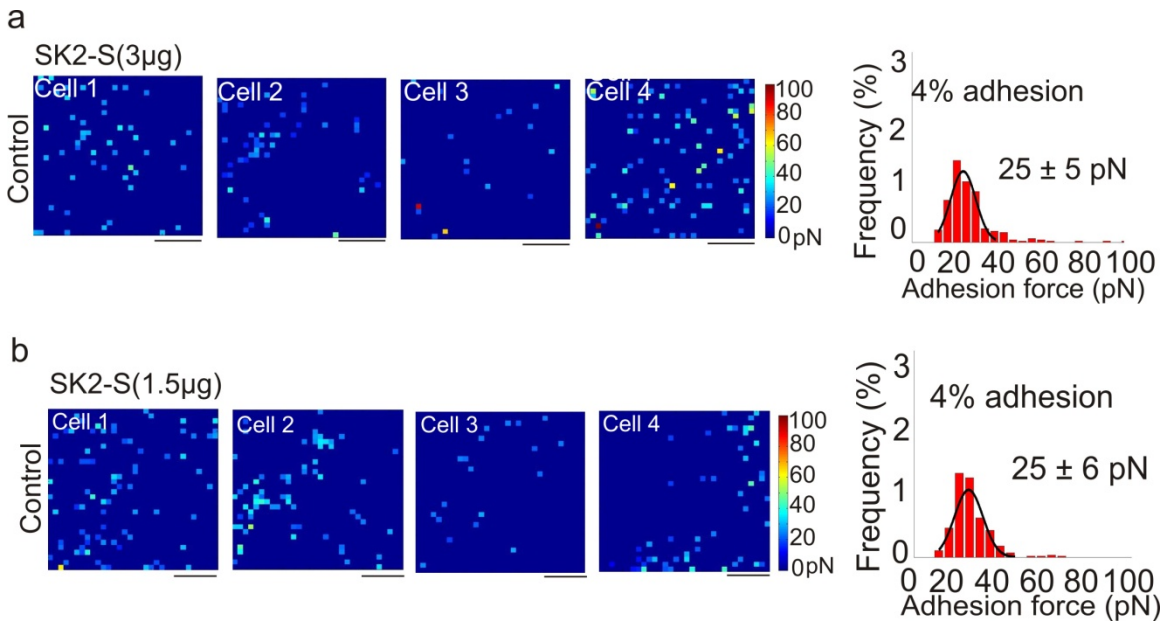

Figure 4.1.1 Baseline experiments on SK2-S: Adhesion map and corresponding histogram of HEK293T cells transfected with (a) 3µg of SK2-S (n=4) and (b) 1.5µg of SK2-S (n=4). Scale bars, 250nm. Color scale as shown

The first step in our aim to study the effect of PKA on SK channel expression was to test the effect of FSK, a PKA activator on the SK channel surface expression. FSK, has previously been demonstrated to decrease the surface expression of SK2 channels (Maciaszek et al., 2012). Addition of 30µM FSK to the SK2-S channel transfected HEK293T cells showed about a 2 fold difference (Fig 4.1.2, p=0.0003, student's t test) in binding events compared to the baseline results and a decrease in mean binding forces (3µg, µ=21±3 pN and 1.5µg, µ=14±3 pN) (Fig 4.1.2). This indicates that the FSK causes a decrease in the surface expression of the SK2-S channels.

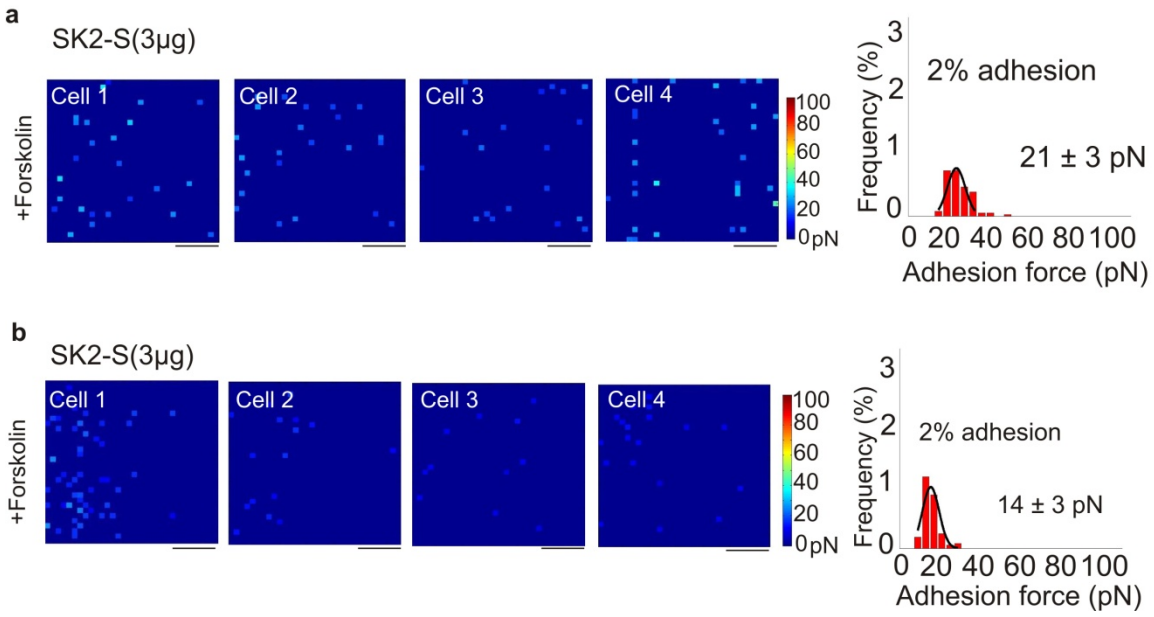

Figure 4.1.2 SK2-S + Forskolin: Adhesion map and corresponding histogram of forskolin addition to HEK293T cells transfected with (a) 3µg of SK2-S (n=4) and (b) 1.5µg of SK2-S (n=4). Scale bars, 250nm. Color scale as shown

In order to determine if the cAMP-dependent PKA causes the decrease in expression of SK channels we tested the effect of a PKA inhibitor KT 5720(1µM) on SK channels. KT 5720 was bath applied to the HEK293T cells and the cells were incubated for 30 minutes. Addition of KT 5720, showed a marked increase in the binding events (3 µg, 15%; 1.5 µg, 10%, n=4). The histogram of the binding forces was best fit as a mixture of two Gaussians (3µg, µ=46±7pN and µ=66±9pN; 1.5 µg, µ=34±6 pN) (Fig 4.1.3). The increased binding events and the bimodal Gaussian distribution of the binding forces suggest the presence of more than one SK2-S channel per recorded site. The mean binding forces are in fact two and three times the mean binding forces found in the baseline

experiments (Fig 4.1.1) indicating that there are two and three SK2-S channels per recording site respectively. SK2-S channels previously thought to exist as single entities now show clustering in the presence of PKA inhibitor. These results suggest the presence of a basal PKA activity that suppresses the surface expression of SK channels in HEK293T cells. Subsequent addition of FSK (30µM) to the KT5720 bathed HEK203T cells did not have any significant effect on the mean binding forces (p=0.3, student's t test) (3µg, µ=44± 9 pN and µ=69± 12pN; 1.5µg, µ=33± 6 pN) (Fig 4.1.4). This substantiates that it is indeed PKA which causes decrease in surface expression of SK2 channels.

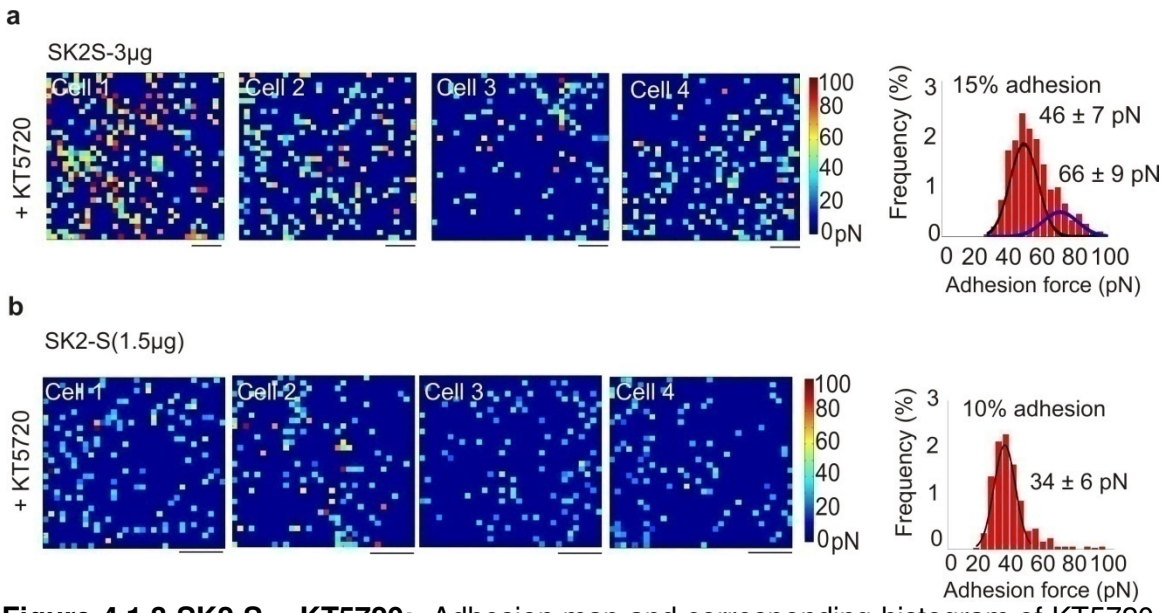

Figure 4.1.3 SK2-S + KT5720: Adhesion map and corresponding histogram of KT5720 addition to HEK293T cells transfected with (a) 3µg of SK2-S (n=4) and (b) 1.5µg of SK2-S (n=4). Scale bars, 250nm. Color scale as shown

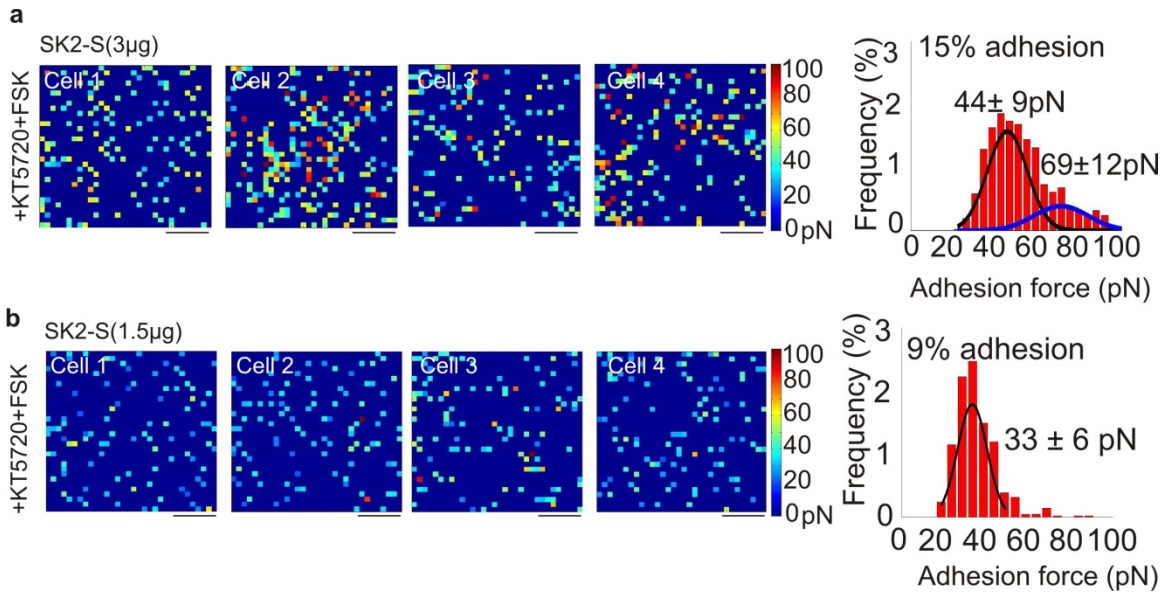

Figure 4.1.4 SK2-S + KT5720+ FSK: Adhesion map and corresponding histogram of KT5720+forskolin addition to HEK293T cells transfected with (a) 3µg of SK2-S (n=4) and (b) 1.5µg of SK2-S (n=4). Scale bars, 250nm. Color scale as shown

Further confirmation that FSK acts via PKA pathway was provided by addition of propranolol. Propranolol is an inverse agonist of β adrenergic receptors which decreases tonic cAMP. The addition of propranolol (10µM) showed an increase in the mean binding percentage (3µg, 7%; 1.5 µg, 9%) (Fig 4.1.5) compared to baseline (Fig 4.1.1) suggesting that. These results taken together with the results obtained from addition of FSK (Fig 4.1.1) and KT 5720 (Fig 4.1.3) prove that the effect of forskolin on SK2 channel expression is through a canonical G_s-PKA signaling pathway

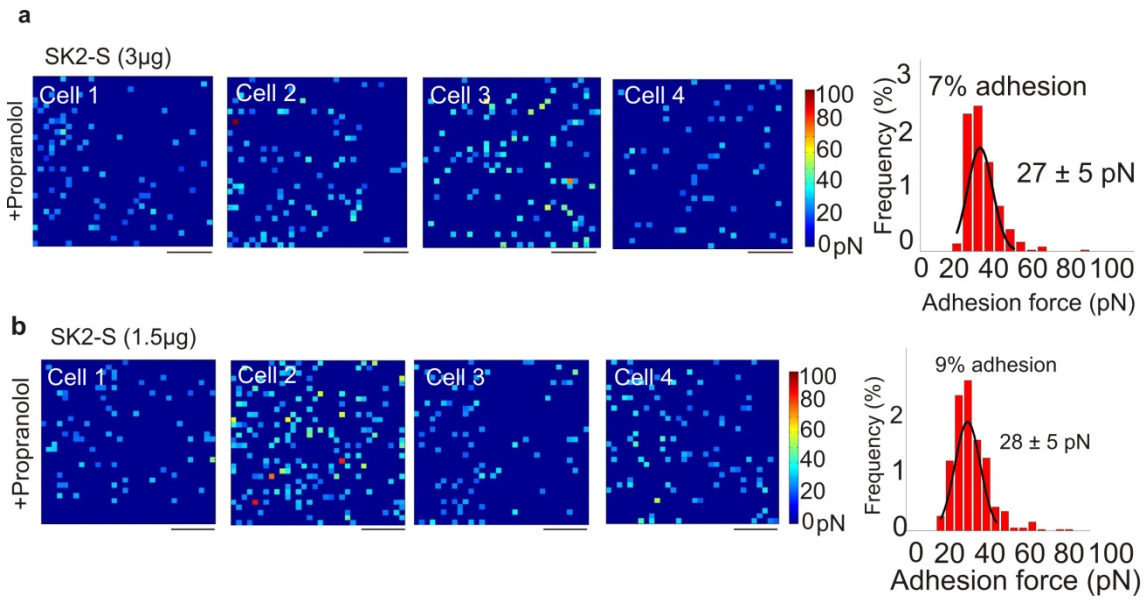

Figure 4.1.5 SK2-S + propranolol: Adhesion map and corresponding histogram of propranolol addition to HEK293T cells transfected with (a) 3µg of SK2-S (n=4) and (b) 1.5µg of SK2-S (n=4). Scale bars, 250nm. Color scale as shown

The intracellular organization of PKA is controlled by A kinase anchoring proteins (AKAPs). AKAPs localize PKA to specific subcellular compartments and thereby increase the specificity of PKA effects. AKAPs are plasma-membrane associated that bind to the regulatory subunit of PKA and localizes it to the plasma membrane. St-Ht31 is a peptide that prevents the association of AKAP with PKA. Addition of St-Ht31 (5 µM) to SK2-S transfected HEK293T cells showed a distinct increase in binding events and mean binding forces (3µg, µ=40±7 pN and µ=57±10 pN; 1.5 µg, µ=37±7 and µ=51±9) (Fig 4.1.6). The histogram of the binding forces was best fit as a mixture two Gaussian distributions. The mean binding forces are in fact two and three times the mean binding forces found in

the control suggesting that there are two and three SK2-S channels per recording site. These results showed that the absence of PKA in the plasma membrane (where the SK2 channels are localized) causes a marked increase in SK2 channel expression.

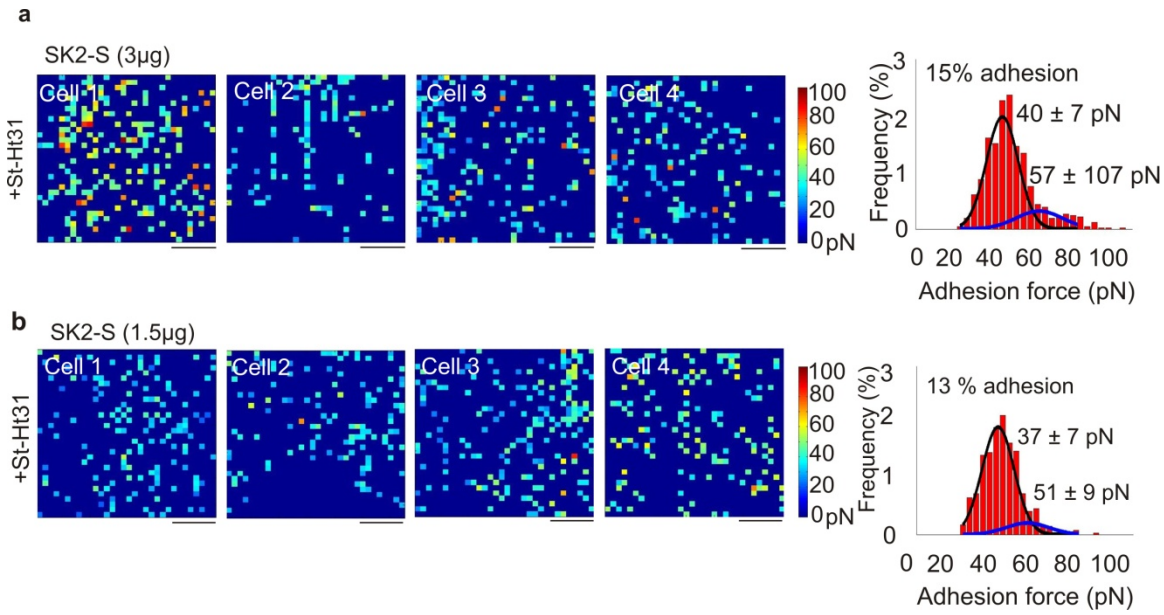

Figure 4.1.6 SK2-S + St-Ht31: Adhesion map and corresponding histogram of St-Ht31 addition to HEK293T cells transfected with (a) 3µg of SK2-S (n=4) and (b) 1.5µg of SK2-S (n=4). Scale bars, 250nm. Color scale as shown

4.2 SK2-L

The long isoform of the SK2 channel (SK2-L) has previously shown to form clusters(Strassmaier et al., 2005). This was observed in baseline experiments between apamin functionalized tips and SK2-L (3µg and 1.5µg, n=4) transected HEK293T cells. The mean binding forces and the binding events (3µg, µ=36±9

pN; 1.5 µg, µ=37±10 pN) (Fig 4.2.1) were significantly greater than when the experiments were performed on HEK293T cells transfected with 3µg and 1.5µg of SK2-S plasmid DNA. This indicates the presence of more than one SK2-L channel per recorded site. On the other hand at very low concentration (0.1 µg) of transfection, the mean binding force was found to be similar to experiments on cells transfected with the SK2-S isoform (data not shown), suggesting that at this very low concentration, single SK2-L channels can be identified (0.1 µg, µ=26±4 pN).

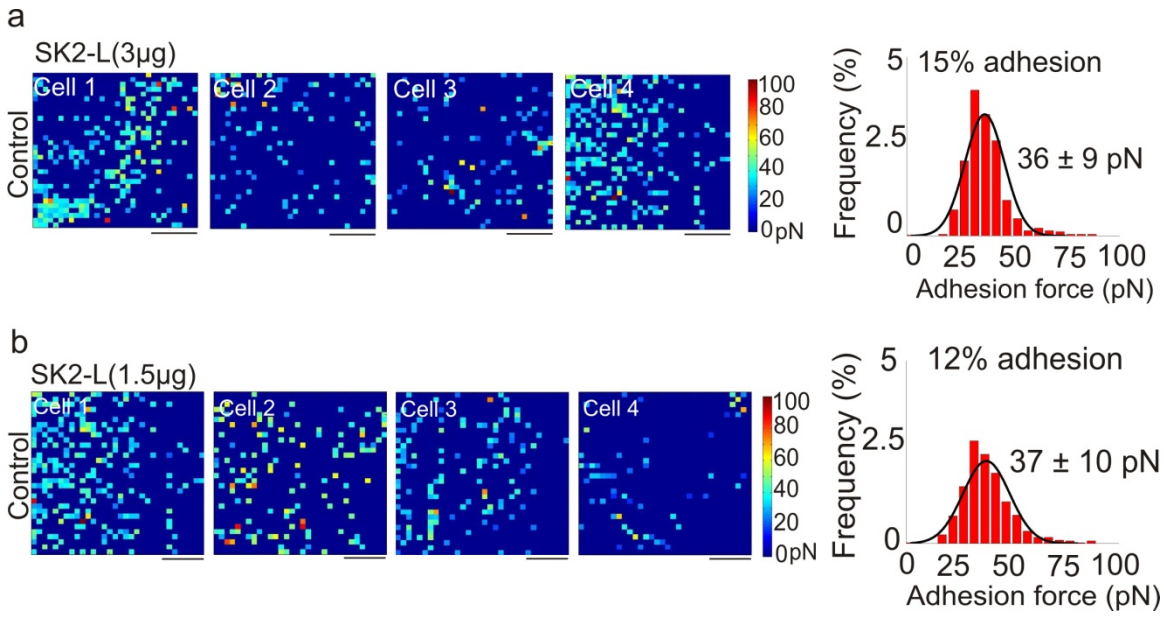

Figure 4.2.1 Baseline experiments on SK2-L: Adhesion map and corresponding histogram of HEK293T cells transfected with (a) 3µg of SK2-L (n=4) and (b) 1.5µg of SK2-L (n=4). Scale bars, 250nm. Color scale as shown

Similar to SK2-S isoform, the effect of forskolin (30 µM) was studied on SK2-L. In the case of SK2-L there were no significant binding forces above baseline upon

addition of forskolin (3µg, µ=12±3 pN; 1.5 µg, µ=13±3 pN) (Fig 4.2.2). As all the forces found were below baseline (<25pN), we find 0% adhesion.

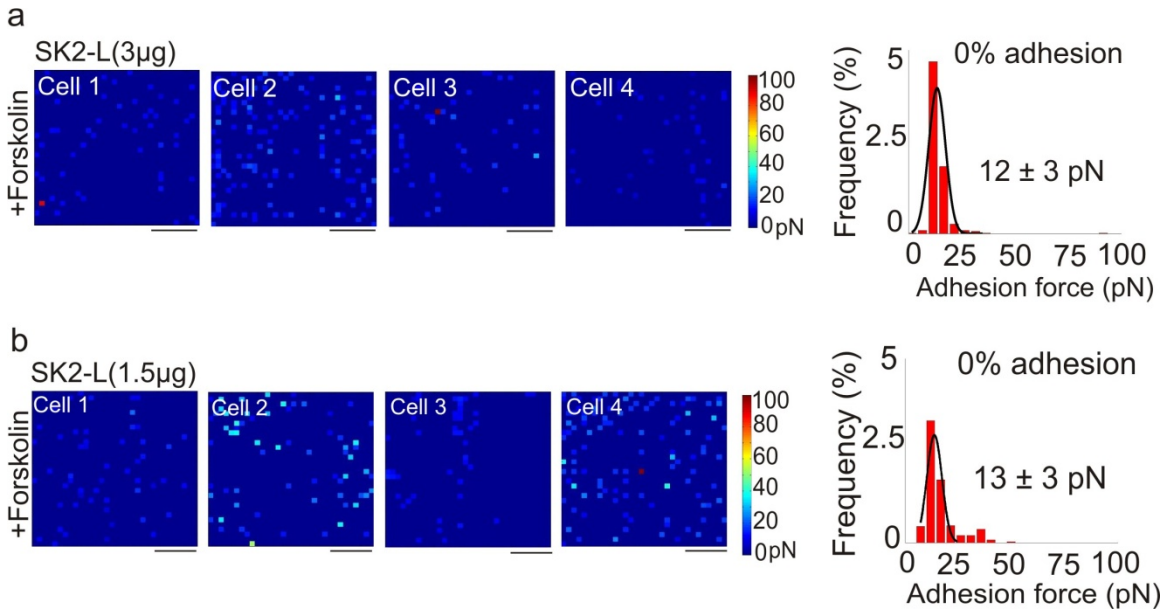

Figure 4.2.2 SK2-L+ Forskolin: Adhesion map and corresponding histogram of forskolin addition to HEK293T cells transfected with (a) 3µg of SK2-L (n=4) and (b) 1.5µg of SK2-L (n=4). Scale bars, 250nm. Color scale as shown

Addition of KT5720 (1µM) to the SK2-L transfected HEK293T cells showed an increase in the mean binding force (3µg, µ=51±11 pN; 1.5 µg, µ=48±10 pN) (Fig 4.2.3) compared to the control. Subsequent addition of forskolin to the KT5720 bathed cells did not significantly change the mean binding force (3µg, µ=54±13 pN; 1.5 µg, µ=44±12 pN) (Fig 4.2.4). Addition of KT5720 caused the clustering of already clustered SK2-L population.

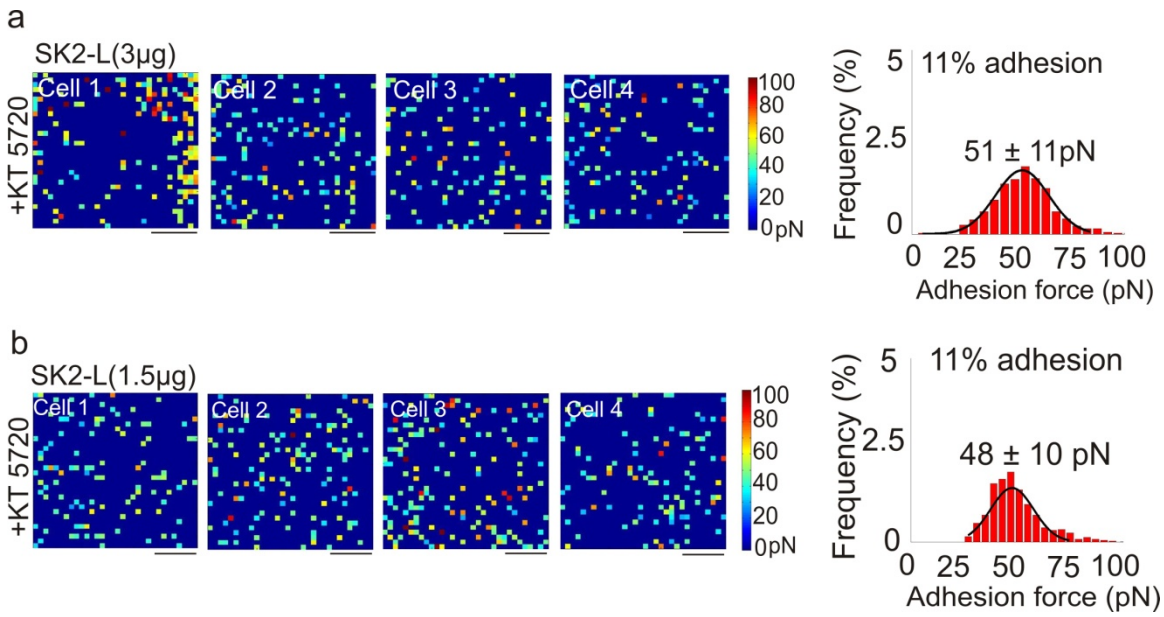

Figure 4.2.3 SK2-L+ KT 5720: Adhesion map and corresponding histogram of KT5720 addition to HEK293T cells transfected with (a) 3µg of SK2-L (n=4) and (b) 1.5µg of SK2-L (n=4). Scale bars, 250nm. Color scale as shown

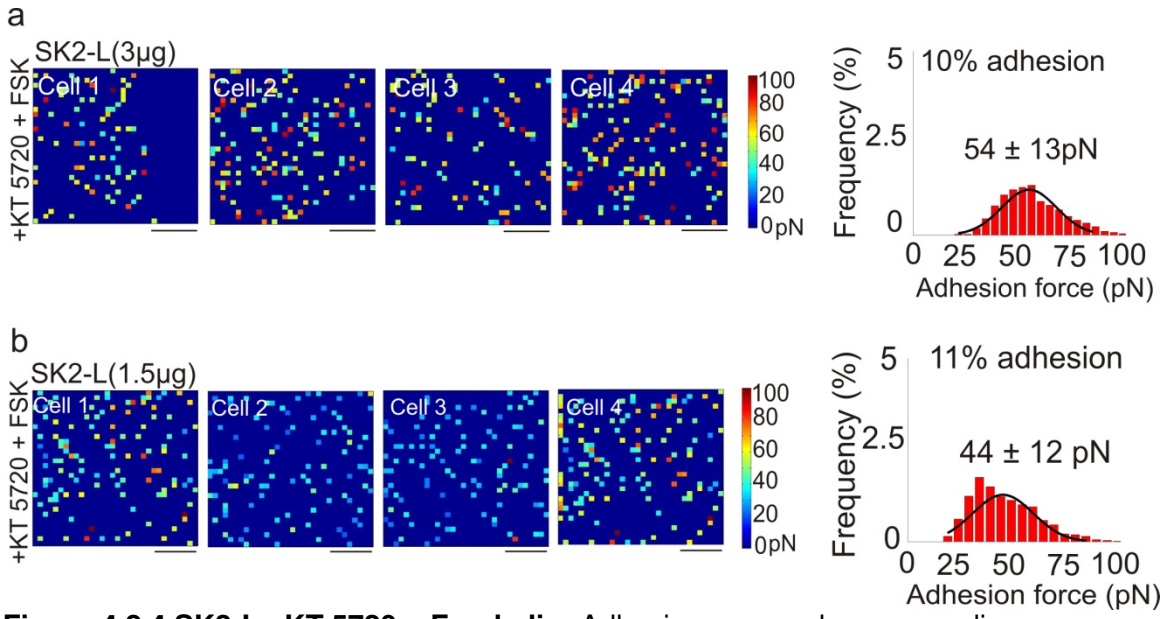

Figure 4.2.4 SK2-L+ KT 5720 + Forskolin: Adhesion map and corresponding histogram of KT5720+forskolin addition to HEK293T cells transfected with (a) 3µg of SK2-L (n=4) and (b) 1.5µg of SK2-L (n=4). Scale bars, 250nm. Color scale as shown

Addition of propranolol (10 µM), resulted in mean binding forces (3µg, µ=37±8 pN; 1.5 µg, µ=33±6 pN) (Fig 4.2.5) similar to control with no significant difference compared to baseline experiments (p=0.6, student's t-test). This suggests that PKA- activated decrease in SK2 channel expression is due to ligand binding to β adrenergic receptors and activation of Gs-PKA pathway.

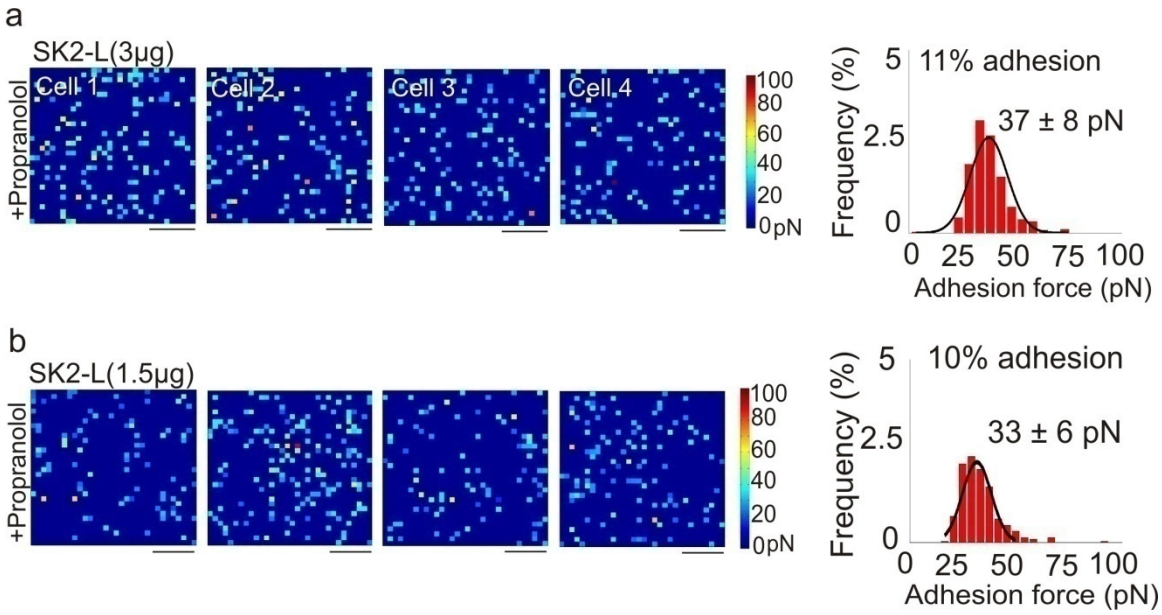

Figure 4.2.5 SK2-L+ Propranolol: Adhesion map and corresponding histogram of propranolol addition to HEK293T cells transfected with (a) 3µg of SK2-L (n=4) and (b) 1.5µg of SK2-L (n=4). Scale bars, 250nm. Color scale as shown

Finally, addition St-Ht31 (5 µM) to SK2-L transfected HEK293T cells showed a distinct increase in binding events and mean binding forces (3µg, µ=35±5 pN and µ=51±10 pN; 1.5 µg, µ=46±6pN and µ=59±7pN) (Fig 4.2.6). The histogram of the binding forces was best fit as a mixture two Gaussian distributions. One of the mean binding forces is similar to the control and the second mean binding force

is approximately twice that of control. This indicates that there are a cluster of two and more than two SK2-L populations.

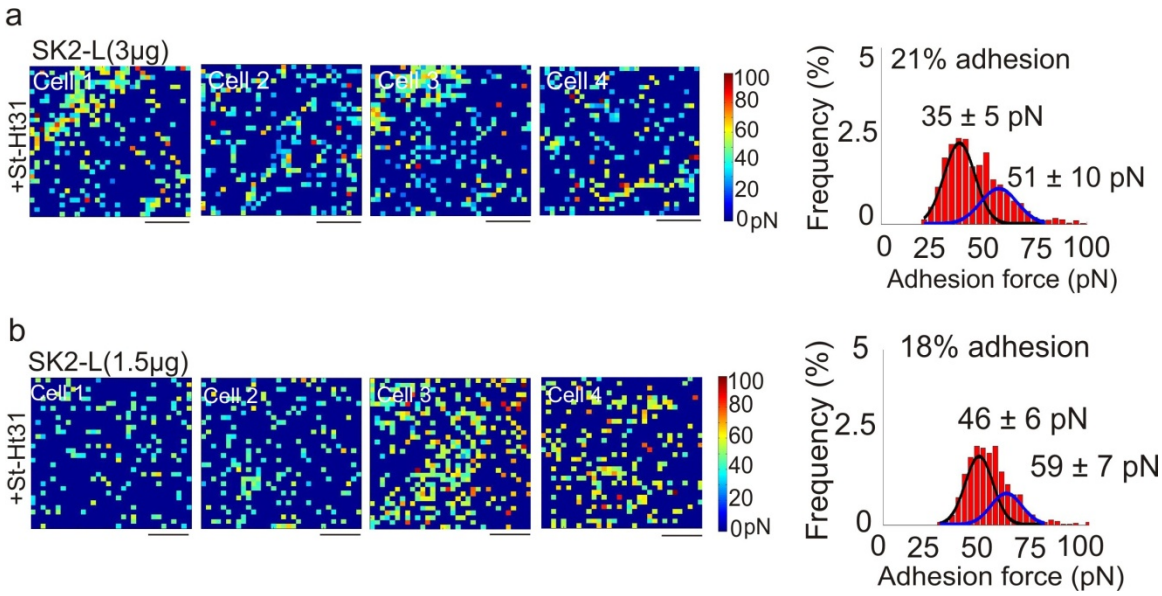

Figure 4.2.6 SK2-L+ St-Ht31: Adhesion map and corresponding histogram of St-Ht31 addition to HEK293T cells transfected with (a) 3µg of SK2-L (n=4) and (b) 1.5µg of SK2-L (n=4). Scale bars, 250nm. Color scale as shown

4.3 Neuromodulation of native SK channels

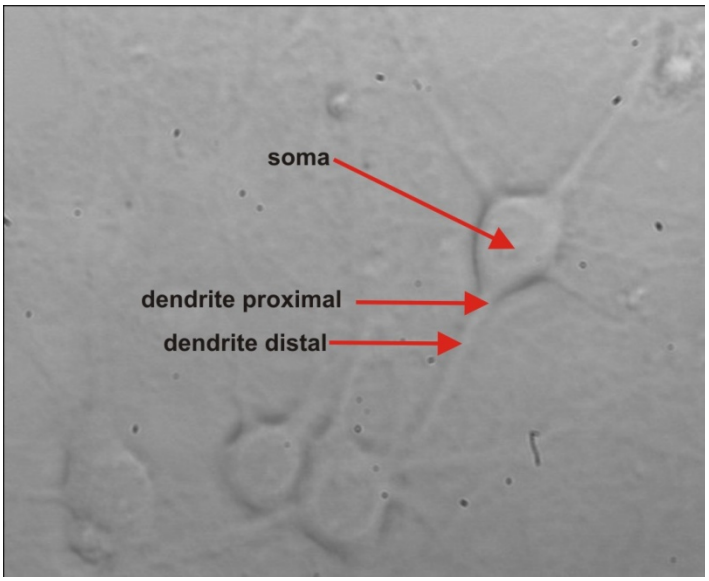

Figure 4.3.1 Optical microscopy image of rat hippocampal neuron: Optical microscopy image of rat hippocampal neuron show the soma, dendrite proximal and dendrite distal.

After establishing the effect of PKA on SK2 channels in HEK293T cells we examined the effect of PKA on native SK2 channels in rat hippocampal neurons. First we performed baseline experiments on neurons with apamin functionalized AFM probes. We probed several 1μm^2 areas focusing on the soma and dendrites proximal and distal. Dendrites proximal to the soma are ~10 μm from the soma and dendrites distal to the soma are ~30μm from the soma. Based on our recordings, we found that SK channel distribution in neurons was highest at the distal dendrites with anadhesion percentage 5% (n=4) and the lowest in the soma (0.7%, n=4) (Fig 4.3.1). The percentage of binding events at the dendrites

proximal was 1% (n=4). The binding force distribution at the dendrites distal did not follow a unimodal distribution but instead were best fit as a mixture of two Gaussians with peaks at 21± 2pN and 32 ± 6pN. These values are similar to those obtained from single and clustered channels in HEK293T cells suggesting that the native SK channels are distributed as single channels or as clusters of two channels in the dendrites of rat hippocampal pyramidal neurons.

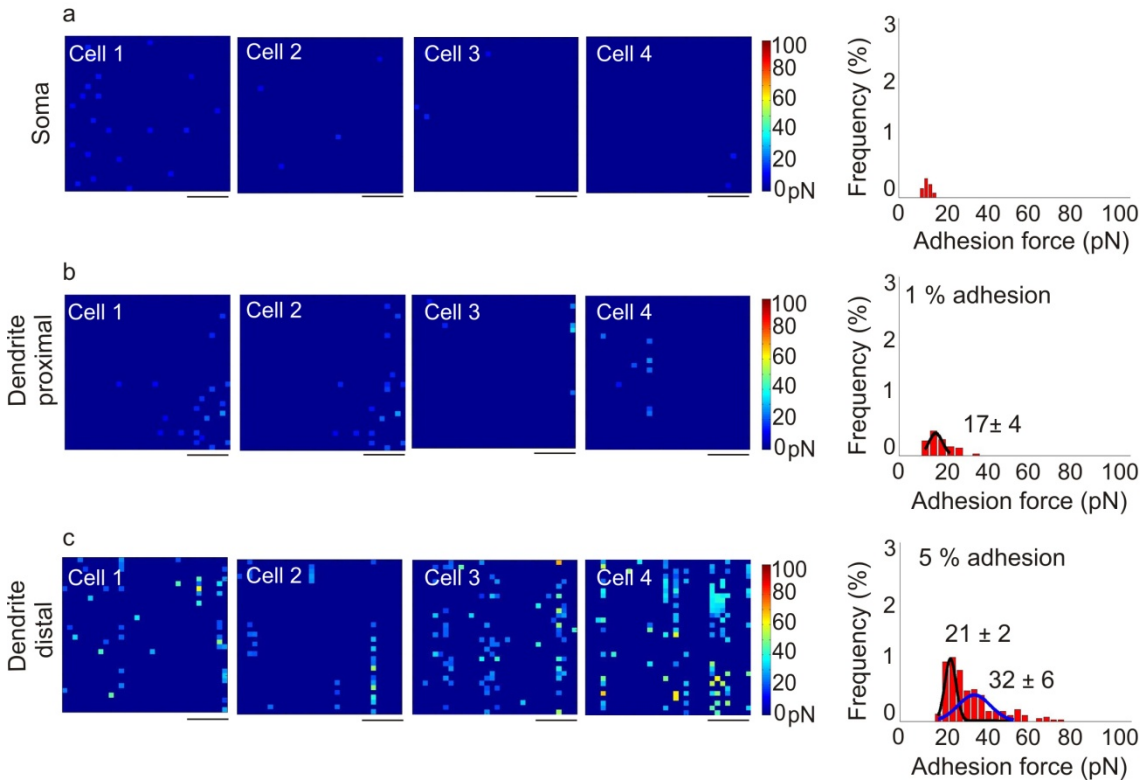

Figure 4.3.2 Localization of SK channels on rat hippocampal neurons: Adhesion map and corresponding histogram showing detected SK2 channels in (a) soma (b) dendrites proximal and (c) dendrites distal. Scale bars, 250nm. Color scale as shown

Lastly, we wanted to test whether tonic PKA activity affects the expression of native SK2 channels in hippocampal pyramidal neurons as in HEK293T cells expressing SK channels. Hence the effect of PKA inhibitor, KT5720 was studied in DIV 16-18 hippocampal pyramidal neurons. Upon addition of KT5720 (1µm) to the neurons, a drastic increase in the binding frequency was found in the soma, dendrite proximal and dendrite distal (n=3) (Figure 4.3.2). The binding force distribution was best fit as a mixture of two Gaussians with mean binding forces at 31± 5pN and 48 ± 11pN, 33 ± 6pN and 49 ± 13pN, 33± 5pN and 47 ±11pN for soma, proximal dendrites and distal dendrites respectively. These results suggest that there is a basal PKA level in the neurons that suppresses the SK2 channel expression, which increases upon PKA inhibition.

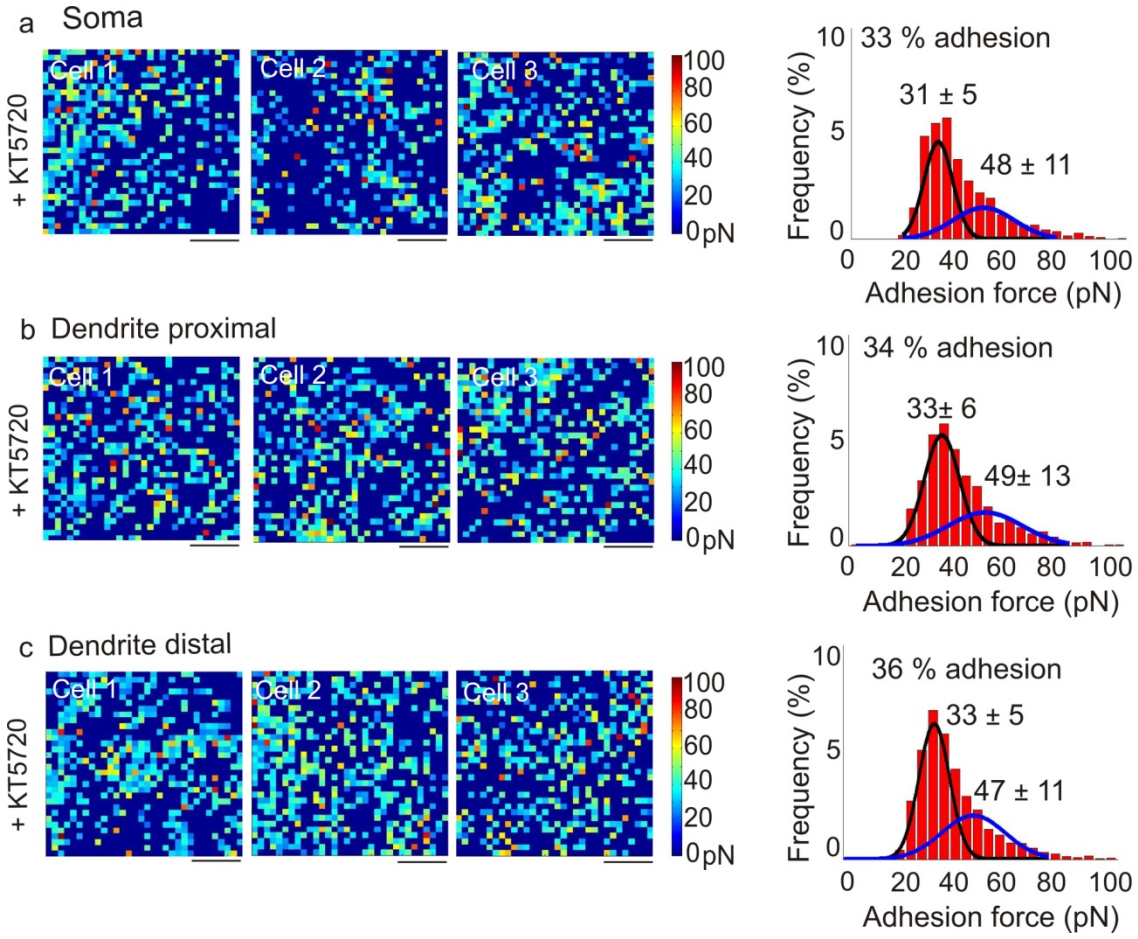

Figure 4.3.3 Neuromodulation of native SK channels in rat hippocampal neurons: Adhesion map and corresponding histogram of KT5720 addition to hippocampal pyramidal neurons in (a) soma (b) proximal dendrites and (c) distal dendrites. Scale bars, 250nm. Color scale as shown

Chapter 5. DISCUSSION

Various neuromodulators have been shown to affect the surface expression of SK2 channels. Addition of forskolin produced a decrease in expression of SK2 channels on HEK293T cell surface. If forskolin modulates SK2 expression through PKA then this effect should be reversed by blocking PKA. In order to determine whether PKA causes the decrease in surface expression of SK2 channels we added KT5720, a PKA inhibitor. The drastic increase in mean binding forces obtained from our recordings indicates that forskolin acts through PKA. Further addition of forskolin did not show any substantial change in mean binding forces suggesting that they are mutually exclusive. These results substantiates that a PKA-dependent mechanism modulates the expression of SK2 channels.

A further proof that FSK acts via PKA pathway was provided by addition of propranolol. Propranolol, a β adrenergic receptor inverse agonist, inhibits cAMP and therefore PKA. Addition of propranolol caused an increase in the adhesive percentage compared to baseline, indicating that PKA activated decrease in SK2 channel expression is due to ligand binding to β adrenergic receptors and activation of Gs protein.

The observation that application of PKA inhibitor KT5720 increases the expression of SK2 channels indicates that PKA is tonically active in the control cells. This tonic PKA keeps the surface expression of the SK2-S to a minimum.

The role of tonic PKA has previously been studied in neurotransmitter release in squid giant synapse(Hilfiker et al., 2001), excitatory postsynaptic potentials in neostriatum(Colwell, 1995), activation of β2 integrins in neutrophils(Chilcoat et al., 2008), catecholamine release from rat chromaffin cells(Koga and Takahashi, 2004). SK2-S previously thought to be non-clustering, formed clusters in the presence of PKA inhibitor suggesting that tonic PKA prevents clustering of SK2-S. However in case of the long isoform, SK2-L, the tonic PKA does not seem to suppress its expression. However the inhibition of PKA causes further clustering of the SK2-L channels. But it is unclear why tonic PKA suppresses SK2-S but not SK2-L and further studies need to be carried out in order to determine the reason for this biased effect of tonic PKA.

AKAPs localize PKA to the plasma membrane which in turn phosphorylates its downstream effectors. By inhibiting the association between AKAP and PKA with inhibitor peptide St-Ht31 we found an increase in SK2 channel expression suggesting that membrane bound PKA modulates the SK2 channel surface expression.

Taken together these results show that there is a tonic PKA which regulates the SK2 channel surface expression and this effect occurs through the canonical Gs-PKA pathway. An agonist activates the β adrenergic receptors via Gs proteins which in turn activates adenylyl cyclase (AC). The AC then generates cAMP which binds to the regulatory subunit of the PKA holoenzyme and activates it. This PKA is then localized to the membrane where the SK channels are located via AKAP79. The PKA then phosphorylates the c terminal region of the SK

channel that has numerous sites for phosphorylation (Faber 2009). The phosphorylation of the SK2 channels causes its internalization and thereby reduces its surface expression.

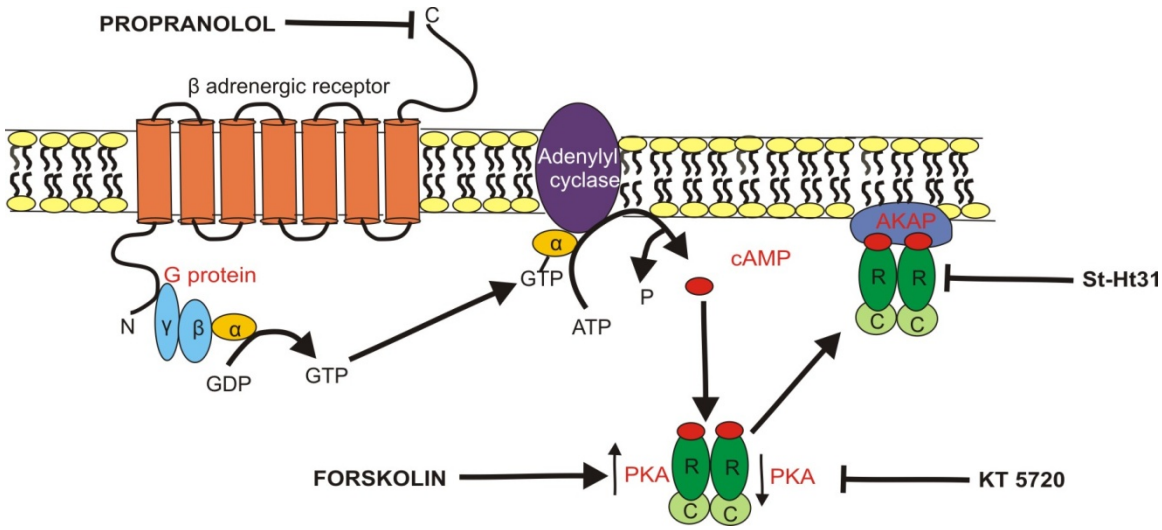

Figure 5.1.2 Signaling pathway: Propranolol an inverse agonist of the β adrenergic receptors reverses the constitutive activity of the receptor. Forskolin is a PKA activator whereas KT5720 is a PKA inhibitor. St-Ht31 is an inhibitory peptide that prevents the association of AKAP with PKA.

Chapter 6. CONCLUSION

By measuring the binding force between the apamin-functionalized AFM cantilever tip and the SK2 channels-expressing cell surface, we studied the effect of cAMP-dependent PKA on the SK2 channel expression. We found that forskolin-mediated reduction in SK2 channel expression is through the cAMP-dependent PKA pathway. While activation of PKA caused the decrease in SK2 channel expression, PKA inhibition caused an opposite effect suggesting the presence of a tonic PKA activity which modulates the surface expression of these channels. Additional experiments validated the presence of tonic PKA. Although these results were established in a heterologous expression system, preliminary results in rat hippocampal neurons expressing native SK2 channels also indicated the presence of tonic PKA activity. Further experiments need to be carried out in rat hippocampal neurons in order to substantiate this.

What is the significance of this tonic PKA-mediated suppression of SK2 channel expression? The reduction in SK2 channel surface expression has previously been shown to contribute to long term potentiation (Lin et al., 2008), synaptic transmission and plasticity (Faber et al., 2008) and its overexpression has been shown to impair learning and memory (Hammond et al., 2006). Perhaps the role of tonic PKA is to modulate one of the above mentioned effects. However further studies need to be carried out to test this possibility.

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